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Influence of IGF-1, TGF-β1 and bFGF on Gene Expression in Cruciate Ligament and Dermal Fibroblasts

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Original article

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Fibroblasts produce extracellular matrix (ECM), secrete proteolytic enzymes, their inhibitors, cytokines and growth factors. Their secretory profile varies depending on the organ from which they originate. We previously described the influence of cartilage interstitial fluid (CIF) on the gene expression of ECM proteins, cytokines, metalloproteases and their inhibitors in synovial membrane cells, dermal (DFs) and cruciate ligament fibroblasts (CTLs). In this work we studied the reaction of rat dermal and cruciate ligament fibroblasts to the most abundant CIF cytokines: basic fibroblast growth factor (bFGF), transforming growth factor-β1 (TGF-β1) and insulin-like growth factor-1 (IGF-1) used in different combinations. The results disclosed several differences in the response of both types of fibroblast to the tested cytokines. For example IGF-1/TGF-β1/bFGF triplet stimulated stronger expression of hyaluronan synthases 1 and 2 (*Has1* and *Has2*) in ligament than dermal fibroblasts. Stronger lubricin (*Prg4)* expression was stimulated by IGF-1/TGF-β1 in cruciate ligament than in dermal fibroblasts. Both combinations of cytokines stimulated *Tgfb1*, tissue inhibitor of metalloproteinases 1 (*Timp1*), inhibited tumor necrosis factor (*Tnf*) and metalloproteinase 3 (*Mmp3*) expression in both types of cells. Because fibroblasts produce different factors in response to the same stimuli, our results provide a better understanding of their role in physiological and pathological processes.

Key words: Cruciate ligament fibroblasts, dermal fibroblasts, cytokines.

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Fibroblasts are well established as the producers of collagen, elastin, proteoglycans and other proteins of the extracellular matrix (ECM) (ÖHLUND *et al.* 2014; SANTI *et al.* 2017). They also secrete proteolytic enzymes and their inhibitors (VAN LINTHOUT *et al*. 2014). Fibroblasts from various organs or tissues differ in secretory activity and function (CHANG *et al*. 2002; NOLTE *et al*. 2008; LINDNER *et al.* 2012; BUECHLER & TURLEY 2017). Cardiac fibroblasts contribute to structural, biochemical, mechanical and electrical properties of the myocardium (CAMELLITI *et al*. 2005). Fibroblasts present in the fasciae play a fundamental role in conveying tension and can dynamically affect mechanical tension, rapidly remodelling their cytoskeletons (LANGEVIN *et al*. 2013; BORDONI & ZANIER 2015). Fibroblasts express toll-like receptors (TLRs), and secrete the panel antimicrobial peptides, cytokines, chemokines and growth factors. Thus, according to their immunological attributes, fibroblasts can also be considered as sentinel cells that recognize pathogens and induce the recruitment of inflammatory cells *via* cytokines and growth factors (BAUTISTA-HERNÁNDEZ *et al.* 2017). There is also compelling evidence that fibroblasts orchestrate the recruitment of and educate other cells to promote cancer growth cells (SANTI *et al.* 2017; SOON *et al.* 2013; RAFFAGHELLO & DAZZI 2015). Synovial fibroblasts are also considered as cells of the innate immune system involved in inflammation and cartilage destruction in rheumatoid arthritis (OSPELT 2017).

We have described the influence of cartilage interstitial fluid (CIF) on the gene expression of ECM proteins, cytokines, metalloproteases and their inhibitors in synovial membrane cells. The observed effects could only be partially imitated by the CIF-like cocktail consisting of factors found in the CIF, i.e. basic fibroblast growth factor (bFGF), insulin-like growth factor-1 (IGF-1), transforming growth factor-β1 (TGF-β1), bone morphogenetic protein-7 (BMP-7), colony stimulating factors for granulocytes and macrophages (G-CSF and M-CSF), leukemia inhibitory factor (LIF) (HYC *at al.* 2016). Moreover, the influence of particular cytokines of CIF differed in the effects exerted by CIF and CIF-like cocktail on the synovial membrane cells (OSIECKA-IWAN *et al.* 2016). Difference in the action of CIF and CIF-like cocktail on the dermal (HYC *at al.* 2017) and cruciate ligament fibroblasts (OSIECKA-IWAN *et al.* 2018) were also noted. In view of these differences, it seemed interesting to compare the reaction of cruciate ligament and dermal fibroblasts towards the three most abundant and active cytokines present in CIF, i.e. bFGF, TGF-β1 and IGF-1 used either separately or in combination on the expression of hyaluronic acid synthases (*Has1, Has2*), ECM proteins (collagen type I – *Col1a2*, versican – *Vcan*, aggrecan – *Acan*, lubricin – *Prg4*), metalloproteinases (*Mmp2, Mmp3*), tissue inhibitors of matrix metalloproteinases (*Timp1, Timp2, Timp3*) and selected cytokines (tumour necrosis factor (*Tnf*), *Tgfb1* and interleukins *Il1b* and *Il6*).

We wanted to examine whether the influence of cytokines produced by chondrocytes and present in the synovial fluid on cells associated with the synovial joint (cruciate ligament fibroblasts) differs from their influence on the same type of cells (dermal fibroblasts) not associated with joints. Because fibroblasts are organ and tissue specific cells involved in multiple activities and can produce different factors in response to the same stimuli, our study is helpful in attaining a better understanding of their role in physiological and pathological processes.

Materials and Methods

Animals

Three-to five day-old Lewis rats of both sexes and ten-to twelve week-old inbred male Lewis rats served as donors of skin and cruciate ligaments, respectively. The animals were obtained from the Animal Unit of the Warsaw Medical University. The study and the methods were approved by the Animal Ethics Committee of the Warsaw Medical University (decision no. 34/2011 of 29th Nov 2011).

Dissection of skin

Three-to five-day old rats were killed by cervical dislocation and put into 10% Antibiotic-Antimycotic Solution (Sigma-Aldrich Chemie, Steinheim, Germany) at 37ºC for 1h. The skin was taken and cut into small fragments.

Dissection of cruciate ligaments

The whole procedure was thoroughly described and illustrated by OSIECKA-IWAN *et al*. (2018). Briefly, knee joints were cut off together with fragments of femur and tibia from 10-12 week old rats. Then all tissues covering the joint together with remnants of joint capsule were removed and both cruciate ligaments were cut off from the menisci and afterwards from the condyles of the femur.

Isolation and culture of cruciate ligament and dermal fibroblasts

The enzyme solution used for cell liberation contained 0.25% collagenase (Type I), 0.05% DNase, 17.5 μM N-p-tosyl-l- lysine chloromethyl ketone and 1% Antibiotic-Antimycotic Solution (Sigma) in RPMI medium (GIBCO, BRL, Paisley, Scotland, UK). Skin fragments were stirred on a magnetic stirrer at 37ºC for 60 min. Ligaments were delicately shaken for 40 min in atmosphere of 5% CO_2 in air at 37°C. After isolation the cells were filtered through a 40-μm mesh nylon filter and seeded into 25 cm^2 flasks (Corning Inc., Corning, NY, USA) in the RPMI medium, supplemented with 10% FBS (Gibco) and 1% of Antibiotic-Antimycotic Solution (Sigma) at a density of 10^6 cells per flask. After the cells reached subconfluency, they were detached with 0.25% trypsin-EDTA (Sigma), rinsed and seeded into 24 well flatbottomed plates (Corning) at a density of $5x10^4$ per well. Control medium contained RPMI supplemented with 2% of FBS and 1% Antibiotic-Antimycotic Solution. Experimental groups contained a combination of cytokines, in concentration found in CIF, IGF-1/TGF-β1(2 ng/ml IGF-1; 0.5 ng/ml TGF-β1) and IGF-1/TGF-β1/bFGF (2 ng/ml IGF-1; 0.5 ng/ml TGF-β1; 2.5 ng/ml bFGF) or only 2.5 ng/ml bFGF. The cells were incubated in a humidified atmosphere of 5% CO_2 in air at 37°C for 24 h. 24 hour incubation of fibroblasts was chosen because this time is needed for the adhesion of isolated cells to the surface of culture dish and for proper extracellular matrix gene expression (LUO *et al.* 2011; ABRAHAMSSON *et al.* 2010). After incubation the total RNA from cultured cells was isolated and the expression of genes encoding hyaluronan synthases (*Has1* and *Has2*), extracellular matrix proteins (collagen type I – *Col1a2*, versican – *Vcan*, aggrecan – *Acan* and lubricin – *Prg4*), ma-

trix metalloproteinases (*Mmp2* and *Mmp3*), tissue inhibitors of metalloproteinases (*Timp1*, *Timp2* and *Timp3*), and cytokines (*Tnf, Il1b* and *Il6, Tgfb1*) was examined.

Total RNA isolation

RNA was isolated with NucleoSpin®RNA II kit (Macherey-Nagel, Duren, Germany), according to manufacturer's protocol. The quantity and quality of the isolated total RNA was evaluated spectrophotometrically using ND-2000-Spectrophotometer NanoDrop 2000 with software for analysis of nucleic acids (Thermo Fisher Scientifc, Wilmington, Delaware, USA).

Reverse transcription

Reverse transcription was performed using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Cheshire, UK), according to the manufacturer's protocol in Eppendorf Mastercycler gradient (10 min at 25°C, 120 min at 37°C and 5 sec. at 85°C). Brie?y, 2 µl of 10x RT buffer, 0.8 µl of 25x dNTP Mix, 2 µl of 10x Random Primers, 1 µl of Multiscribe Reverse Transcriptase, 4.2 µl of nuclease-free water and 10 µl of mRNA (0.5 µg) per one reaction. cDNA samples were stored at -20°C.

Real-time PCR

Real-time PCR was performed in the ABIPRISM 7500 (Applied Biosystems) using 96-well optical plates. Each sample was run in triplicate and was supplied with an endogenous control (Rat *Gapdh* endogenous control Rn01775763_g1). For gene expression analysis, proper TaqMan expression assays was used. (Rn00597231_m1 for *Has1,* Rn00565774_m1 for *Has2,* Rn01526721_m1 for C*ol1a2*, Rn01493763_m1 for *Vcan*, Rn00573424_m1 for *Acan*, Rn01490812_m1 for *Prg4*, Rn01538167 for *Mmp2*, Rn00591740_m1 for *Mmp3*, Rn00587558_m1 for *Timp1*, Rn00573232_m1 for *Timp2*, Rn00441826_m1 for *Timp3*, Rn00572010_m1 for *Tgfb1*, Rn99999017_m1 for *Tnf,* Rn00580432_m1 for *Il1b* and Rn01410330_m1 for *Il6*). All probes were stained with FAM (Applied Biosystems). Reactions were run in 25 µl with TaqMan Universal Master Mix, appropriate primer set, MGB probe and 50 ng of cDNA template. Universal thermal conditions, 10 min at 95°C, 40 cycles of 15 sec at 95°C and 1 min at 60°C, were used. Data analysis was done with sequence detection software version 1.2 (Applied Biosystems). Relative expression was calculated against the reference gene, *Gapdh*. Analysis was conducted as a relative quantification study, using unstimulated cruciate ligaments or dermal fibroblasts (control) gene expression as a calibrator (value = 1).

Statistical analysis

Data were analyzed by a Mann-Whitney U test (Statistica12 software; StatSoft Polska, Krakow, Poland). In the first analysis, the expression of mRNA for each particular gene in control fibroblasts (unstimulated) was compared with mRNA expression in experimental fibroblasts (different combination of cytokine stimulation). In the second analysis, the expression of mRNA for each particular gene in cruciate ligament fibroblasts was compared with mRNA expression in dermal fibroblasts. In each group contained n=8. P.05 was considered to indicate a statistically significant difference (LIVAK & SCHMITTGEN 2001).

Results

In cruciate ligament, fibroblasts (CLFs) IGF-/TGF-β1/bFGF stimulated the expression of genes encoding *Has1, Has2*, *Col1a2*, *Vcan*, *Acan*, *Prg4*, *Timp1*, *Tgfb1*, *Il1b* and *Il6*. The expression of *Mmp3*, *Timp3* and *Tnf* was inhibited and the expression of *Mmp2*, *Timp*2 was unchanged. The same combination of growth factors stimulated *Has1, Has2, Col1a2, Vcan, Mmp2, Timp1, Timp2, Timp3, Tgfb1* and *Il6*, inhibited *Mmp3* and *Tnf* and had no effect on the expression of *Acan*, *Prg4* and *Il1b* in dermal fibroblasts (DFs).

Stimulation of fibroblasts with IGF-1/lTGF-β1 increased the expression of *Has1, Has2, Acan, Prg4, Timp1, Tgfb1*and *Il6*, decreased the expression of *Mmp3, Timp2, Timp3, Tnf* and *Il1b* and did not change the expression of *Col1a2*, *Vcan* and *Mmp2* in CLFs. The same cytokines stimulated the expression of *Has1, Has2, Col1a2, Prg4, Timp1* and *Tgfb1*, inhibited the expression of *Mmp3* and *Tnf* and had no effect on the expression of *Vcan*, *Acan, Mmp2, Timp2, Timp3, Il1b* and *Il6* in DFs.

Added separately bFGF increased the mRNA level for *Col1a2, Vcan, Acan, Timp1, Timp2, Timp3, Tgfb1, Il1b* and *Il6,* had no effect on *Has1, Has2, Prg4, Mmp2, Mmp3* and *Tnf* in CLFs. In dermal fibroblasts, bFGF stimulated the expression of *Has2, Col1a2, Vcan, Acan, Timp1, Timp2, Timp3, Tgfb1* and *Il1b*, inhibited *Prg4* and had no effect on *HasS1, Mmp2, Mmp3, Tnf* and *Il6* expression (Table 1, Fig. 1-3).

Although IGF-/TGF-β1/bFGF stimulated the expression of *Has1, Has2, Vcan, Timp1, Tgfb1* and *Il6* in both types of fibroblasts, the expression of these genes in cruciate ligament fibroblasts was statistically higher than in dermal fibroblasts. Moreover, expression of *Acan* and *Prg4* was stimulated only in cruciate ligament fibroblasts. IGF-/TGF-β1/bFGF exerted the same effect on the

Table 1

Influence of IGF-1, TGF-β1 and bFGF on gene expression in dermal and cruciate ligament fibroblasts

Gene	Dermal fibroblasts			Cruciate ligament fibroblasts		
	IGF-1/ TGF-β1/ bFGF	IGF-1/ TGF-β1	bFGF	IGF-1/ TGF-β1/ bFGF	IGF-1/ TGF-β1	bFGF
HAS1	+	+	no effect	++	++	no effect
HAS2	+	+	+	++	++	no effect
Collagen I	+	+	+	+	no effect	+
Versican	+	no effect	+	++	no effect	+
Aggrecan	no effect	no effect	+	+	+	+
Lubricin	no effect	+	–	+	++	no effect
MMP2	+	no effect	no effect	no effect	no effect	no effect
MMP3	–	–	no effect	–	–	no effect
TIMP1	+	+	+	++	++	++
TIMP2	+	no effect	+	no effect	–	+
TIMP3	+	no effect	+	–	–	+
TGF-β1	+	+	+	++	+	+
TNF	–	–	no effect	–	–	no effect
IL-1β	no effect	no effect	+	+	–	+
IL-6	+	no effect	no effect	++	+	+

Minus (–) – inhibition of gene expression, plus (+) – stimulation of gene expression. Double plus (++) – stimulation of gene expression by the same factor statistically stronger in cruciate ligament fibroblasts than in dermal fibroblasts (+) (Mann-Whitney test, $P<0.05$).

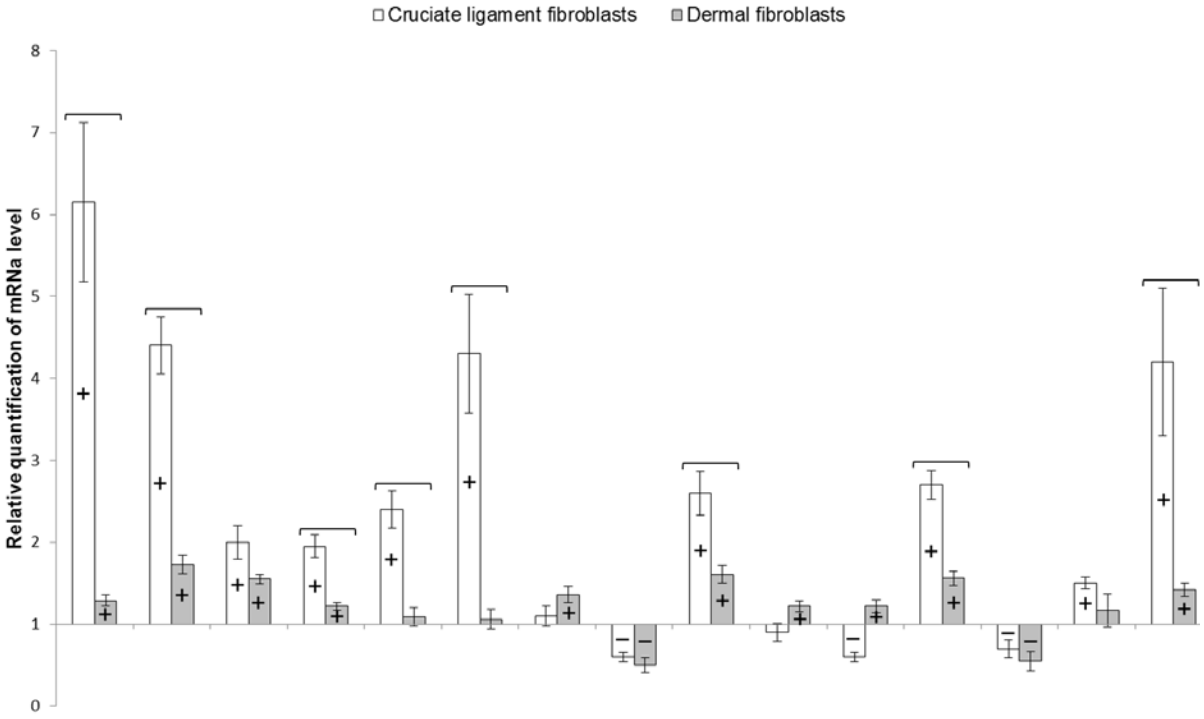

Fig. 1. Mean values ± SE of hyaluronan synthases, extracellular matrix proteins, MMPs, TIMPs and cytokine mRNA levels in cruciate ligament and dermal fibroblast after 24 h of incubation with IGF-1/TGF-β1/bFGF measured by real time PCR. In each group n=8. Statistically significant differences ($P<0.05$) in the expression of particular genes in relation to control are marked with pluses (stimulation) or minuses (inhibition). Statistically significant differences between the influence of IGF-1/TGF-β1/bFGF on cruciate ligament and dermal fibroblasts are joined by brackets.
Has1 and *2*, hyaluronan synthases 1 and 2; *Col1a2*, collagen 1; *Vcan*, versican; *Acan*, aggrecan; *Prg4*, lubricin; *Mmp2* and *3,* matrix metalloproteinase 2 and 3; *Timp1-3*, tissue inhibitors of metalloproteinasas 1-3; *Tgfb1*, transforming growth factor-β1; *Tnf*, tumor necrosis factor; *Il1b*, interleukin-1β; *Il6*, interleukin-6; IGF-1, insulin-like growth factor-1; bFGF, basic fibroblast growth factor.

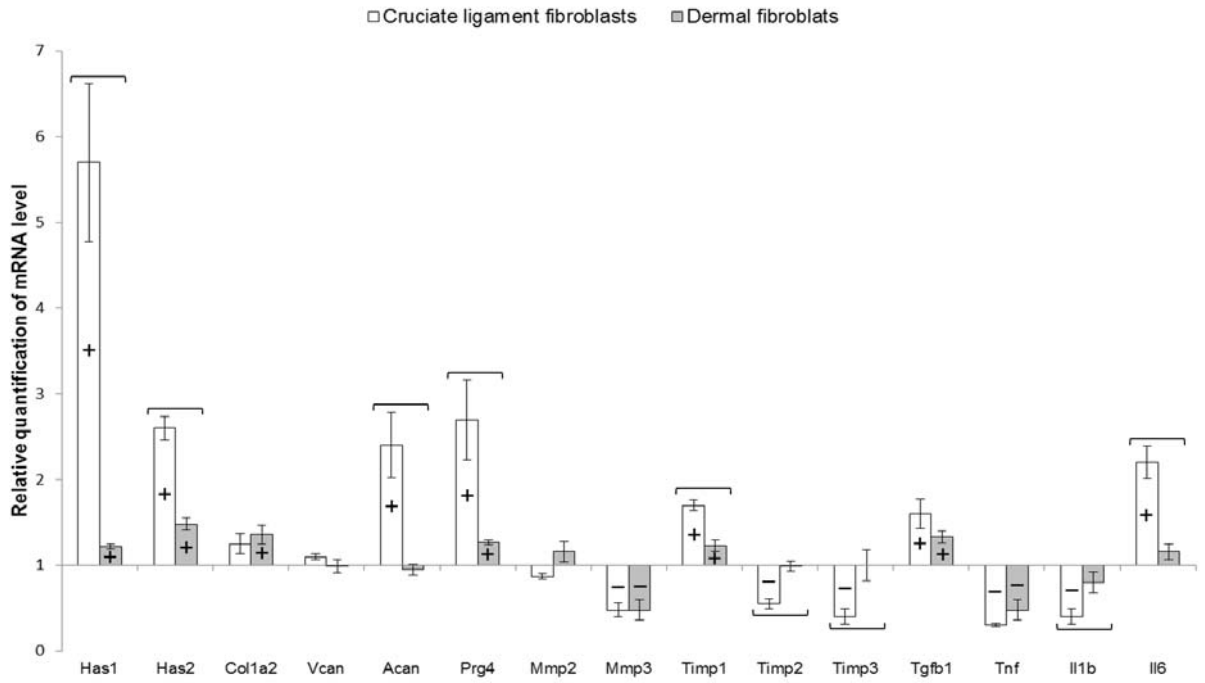

Fig. 2. Mean values ± SE of hyaluronan synthases, extracellular matrix proteins, MMPs, TIMPs and cytokine mRNA levels in cruciate ligament and dermal fibroblast after 24 h of incubation with IGF-1/TGF-β1 measured by real time PCR. In each group n=8. Statistically significant differences (P<0.05) in the expression of particular genes in relation to control are marked with pluses (stimulation) or minuses (inhibition). Statistically significant differences between the influence of IGF-1/TGF-β1 on cruciate ligament and dermal fibroblasts are joined by brackets. For abbreviations see Fig. 1.

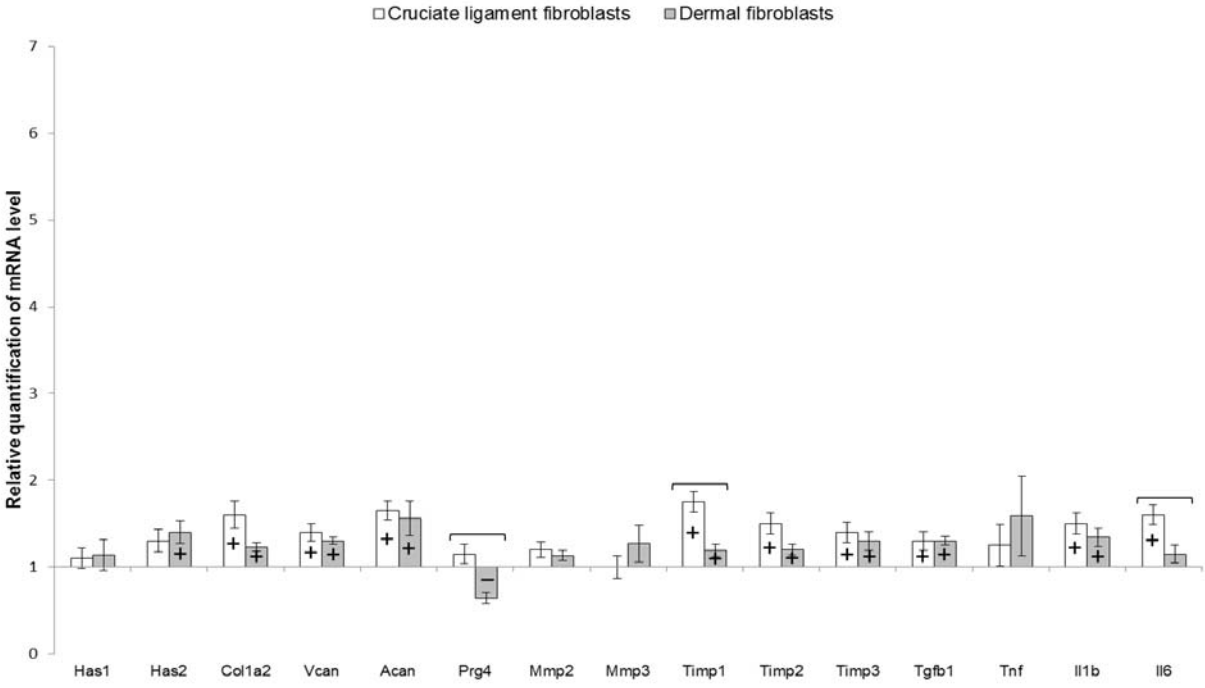

Fig. 3. Mean values ± SE of hyaluronan synthases, extracellular matrix proteins, MMPs, TIMPs and cytokine mRNA levels in cruciate ligament and dermal fibroblast after 24 h of incubation with bFGF measured by real time PCR. In each group n=8. Statistically significant differences (P<0.05) in the expression of particular genes in relation to control are marked with pluses (stimulation) or minuses (inhibition). Statistically significant differences between the influence of bFGF on cruciate ligament and dermal fibroblasts are joined by brackets. For abbreviations see Fig. 1.

expression of *Col1a2* (stimulation), *Mmp3* and *Tnf* (inhibition) in both types of fibroblasts (Fig. 1).

IGF-/TGF-β1 increased the mRNA level for *Has1, Has2, Prg4* and *Timp1* in both types of fibroblasts, but the effect was statistically stronger in ligament fibroblasts. *Acan* and *Il6* expression was stimulated and *Timp2, Timp3* and *Il1b* expression was inhibited only in cruciate ligament fibroblasts (Table 1).

IGF-/TGF-β1 exerted the same effect on the expression of *Tgfb1* (stimulation), *Mmp3* and *Tnf* (inhibition) (Fig. 2).

bFGF stimulated the expression of *Col1a2, Vcan, Acan, Timp1, Timp2, Timp3, Tgfb1* and *Il1b* in both types of fibroblasts, but the effect was statistically stronger only for *Timp1* in ligament fibroblasts. The expression of *Il6* was stimulated only in cruciate ligament fibroblasts and lubricin expression was inhibited only in dermal fibroblasts (Fig. 3).

Discussion

Analysis of influence exerted by various cytokines is usually limited to the study of a single factor. The present study was driven by the observation that articular cartilage releases under pressure several factors (cartilage interstitial fluid – CIF) which exert a profound influence on the synovial membrane, and dermal and cruciate ligament fibroblasts. It was also found that factors present in CIF used jointly could have synergistic or antagonistic effects. For example, TGF-β1/IGF-1 stimulated *Acan* expression in the synovial membrane cells while TGF-β1/IGF-1/bFGF had no effect (OSIECKA-IWAN *et al.* 2016). The purpose of this work was to analyze the influence of the three most abundant factors (IGF-1, TGF-β1, bFGF) in CIF which exerted a clear-cut effect on the synovial membrane (OSIECKA-IWAN *et al.* 2016) in other, simpler systems consisting of isolated dermal and cruciate ligament fibroblasts.

The effect of the IGF-1/TGF-β1 pair and bFGF alone was also studied to see whether its presence influenced triplet IGF-1/TGF-β1/bFGF performance.

Stimulation by IGF-1/TGF-β1/bFGF and IGF-1/TGFβ1 increased the gene expression of *Has1* and *Has2* in both dermal and cruciate ligament fibroblasts, but was significantly stronger in the latter. Similar effects on *HASs* were observed in skin fibroblast after TGF-β1 and IGF-1 treatment (KURODA *et al.* 2001; NAGAOKA *et al.* 2015). Stimulation of *HASs* expression by TGF-β1 was also noted in fibroblast-like synoviocytes (STUHLMEIER & POLLASCHEK 2004).

Proteins characteristic for connective tissue proper collagen type I and versican were stimulated only by IGF-1/ TGF-β1/bFGF and bFGF in both types of fibroblasts. The stimulatory effect of *Tgfb1* on *Vcan* expression in lung fibroblasts was observed by VENKATESAN *et al*. (2002) but, contrary to our results, bFGF did not alter versican expression in dermal fibroblasts (TAN *et al.* 1993). In cruciate ligament fibroblasts TGF-β1 and bFGF increased the production of collagenous and non-collagenous extracellular matrix proteins (CHENG *et al.* 2009). Expression of lubricin and aggrecan, proteins present in the joint cavity and articular cartilage, was stimulated by IGF-1/ TGF-β1/bFGF and IGF-1/TGF-β1 in cruciate ligament fibroblasts. In dermal fibroblasts only the expression of lubricin was increased by IGF-1/TGF-β1 treatment. Our results are in agreement with those of FOX *et al.* (2010), who found that IGF-1/TGF-β1 increased the expression of aggrecan in equine synovial fibroblasts. Similarly, increased lubricin expression was observed in synovial fibroblasts after TGF-β1 treatment (BARRIENTOS *et al*. 2010).

Stimulation with IGF-1/TGF-β1/bFGF increased the expression of *Mmp2* only in dermal fibroblasts. Since TGF-β1 was found to induce an increase in the amount of *MMP2* in cruciate ligament fibroblasts (WANG *et al.* 2011) and IGF-1 increased the *MMP2* expression in lung fibroblasts (LI *et al.* 2015), our results are in agreement with these previous studies. The expression of *Mmp3* was inhibited by both combinations of factors in cruciate ligament and dermal fibroblasts. This result is in good agreement with our previous experiments, in which TGF-β1 and a combination of IGF-1/ TGF-β1/bFGF inhibited *Mmp3* gene expression in synovial membrane cells (HYC *et al*. 2011), but is opposite to BARRIENTOS *et al*. (2008) who showed that TGF-β1 increased the expression of *MMP3* in dermal fibroblast. Inhibition of expression of *Mmp3* was observed with a simultaneous increase of *Timp1* expression which was stimulated in all experiments in dermal and cruciate ligament fibroblasts, but strongly in the latter. Similar results on *TIMP1* expression were obtained for myofibroblasts (YASUI *et al.* 2004).

The expression of anti-inflammatory cytokine – *Tgf1b* was increased in all tested groups. TGF-β1 can induce its own gene expression in fibroblasts (VAN OBBERGHEN-SCHILLING *et al*. 1988; KIM *et al.* 1990; PIEK *et al*. 2001), thus the rise in its expression in both types of fibroblasts under the influence of TGF-β1 present in a pair or triplet of cytokines was to be expected. Its expression was also raised by bFGF treatment, agreeing with the observation that bFGF may potentiate the release of TGF-β1 by macrophages (YUM *et al*. 2011) or its expression in rat glioma cells and astrocytes

(DHANDAPANI *et al*. 2007). TGF-β1 may cause differentiation of fibroblasts into myofibroblasts (TOMASEK *et al.* 2002) or induce smooth muscle gene expression (KAWAI-KOWASE *et al.* 2004), the last effect is antagonized by bFGF. *Tnf* expression remained unchanged in both types of fibroblasts under the influence of bFGF and was down-regulated by IGF-1/TGF-β1 and IGF-1/TGF-β1/bFGF. In bone marrow stromal cells IGF-1 inhibited *Tnf* gene expression (GUO *et al.* 2014). Similarly, IGF-1 attenuated release of TNF from glial cells (DODGE *et al.* 2008). TGF-ß1 did not change the expression of *Tnf* in rat synovial membrane incubated in vitro (HYC *et al.* 2011), but decreased it in human fibroblasts (TURNER *et al.* 1991). In our experiments, therefore, IGF-1 was probably mainly responsible for the observed inhibitory effect on *Tnf* expression, but the contribution of TGF-β1 cannot be excluded. Only stimulation with bFGF increased the expression of *Il1b* and solely treatment with IGF-1/TGF-β1/bFGF stimulated *Il6* expression in dermal fibroblasts. However, in cruciate ligament fibroblasts, the expression of *Il1b* was inhibited by IGF-1/TGF-β1 and stimulated in other experiments, and *Il6* stimulation was increased in all tested groups.

Since bFGF used alone stimulated *Il1b* expression, it seems that bFGF has a dominant position and its presence overcomes the inhibitory effect of the IGF-1/TGF-β1 pair. In other studies IGF-1 inhibited protein production and gene expression of *Il1b* and *Il6* in bone marrow stromal cells (GUO *et al.* 2014; HIDESHIMA *et al.* 2005) and TGF-ß1 inhibited *IL-1β* and stimulated *IL-6* expression by human fibroblasts (TURNER *et al.* 1991; EICKELBERG *et al.* 1999; SEONG *et al.* 2009). IGF-1/TGF-β1 and bFGF decreased the mRNA levels of *Il1b* and had no effect on rat synovial membrane cells (HYC *et al.* 2011).

Development of cruciate ligaments is related to the formation of other joint structures. The long bones of the vertebrate appendicular skeleton arise from initially continuous condensations of mesenchymal cells that subsequently differentiate, segment and cavitate to form discrete elements separated by synovial joints (HARTMANN & TABIN 2001). Cartilage templates of neighbour bones become separated through development of a non-cartilaginous region known as the interzone. The differentiation of interzone mesenchymal cells may continue until components like menisci, synovial membrane or ligaments in knee joints are produced and the joint forms a "mini organ" of differentiated tissues (ANDERSEN & BRO-RASMUSSEN 1961). Thus, the stronger response of CLFs than DF to cytokines stimulating expression of aggrecan and lubricin, proteins produced by chondrocytes, may be explained by their developmental origin. In torn anterior cruciate ligaments (ACLs), lubricin was generally found as a discrete layer covering the torn surface. Lubricin was also found on the native surfaces of intact ACLs and on their torn edges. Thus, it may interfere with the integrative healing process needed for ACL repair (ZHANG *et al.* 2011). The presence of aggrecan in fibrocartilaginous ligaments was also reported previously (MILZ *et al.* 2006). Moreover, cytokines used in combinations could regulate the production of pro- and anti-inflammatory cytokines by fibroblasts. They inhibited the expression of *Tnf* – a potent pro-inflammatory cytokine, and stimulated the expression of *Tgfb1* – an important anti-inflammatory cytokine. It seems also significant that the tested cytokines had no or an inhibitory influence on *Mmp3* expression but stimulated the expression of *Timp1* in both types of fibroblasts as if to give them double protection against proteolysis. This suggests that cytokines studied in this work could limit the inflammatory process in cruciate ligaments. These results could be useful for the choice of proper composition of growth factor cocktails for stimulation of damaged connective tissue healing.

Studies on the reaction of cruciate ligament fibroblasts to growth factors gained additional perspective because of the use of platelet-rich plasma (PRP) injection in the treatment of synoviol joints (KABIRI *et al*. 2014). Because PRP contains i.e. TGF-β1, IGF-1 and bFGF, our results could be useful for a better understating of fibroblast response to PRP injections.

Author Contributions

Research concept and design: A.H., A.I.; Collection and/or assembly of data: A.H., M.Ż., A.I.; Data analysis and interpretation: A.H., A.I.; Writing the article: A.H., A.I.; Critical revision of the article: A.H., A.I.; Final approval of article: A.H., A.I.

Conflict of Interest

The authors declare no conflict of interest.

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